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(57) Abstract

A cryopreservation procedure that yields high percentages of normal births after rapid thawing of frozen embryos and implantation into recipient females. The techniques have been successfully used to cryopreserve mouse, rabbit and bovine embryos. *In vitro* success rates ranged from 75 to 90 % survival to the mature blastocyst stage for mice and rabbits. *In vivo* rates for implantation and survival of embryos ranged from 15 to 30 % and 40 to 50 % for mice and rabbits, respectively. Embryos are obtained by flushing the oviduct and uterus of donor females following prescribed periods post hCG using rehydration/recovery medium. These are equilibrated with equilibration medium for between five and ten minutes, preferably for seven minutes. Artificial insemination straws are prepared containing layers of vitrification medium separated by air barriers, loaded with the embryos in as small a volume of equilibration medium as possible with additional vitrification medium added after the final air barrier. Sealant/identification rods are placed on the open end of the straws which are then vitrified by progressive immersion into liquid nitrogen. Embryos are recovered by removing the straw from the liquid nitrogen and immediately immersing it into a room temperature water bath for five to ten seconds, unsealing both ends of the straw, and emptying the contents into a dish of dilution medium for five minutes. Following dilution, rehydration occurs when the embryos are placed into a volume of rehydration/recovery medium for fifteen minutes. After the proper incubation time, the embryos can be implanted into recipient females.

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METHOD AND COMPOSITIONS FOR ONE-STEP CRYOPRESERVATION OF EMBRYOS

Background of the Invention

This is generally in the area of cryopreservation of embryos.

As first developed, embryos were cryopreserved in isotonic medium with cryoprotectant(s) added in solution. A cryoprotectant is a solute or solution that is added to medium to protect intracellular organelles during long-term cryopreservation, usually in liquid nitrogen. General freezing procedures were improved upon by allowing embryo pre-equilibration periods with varying concentrations of cryoprotectants. Embryos, recovered and temporarily incubated in isotonic modified phosphate buffered saline and/or culture media, become exposed to increasing concentrations of cryoprotectant in stepwise manner, allowing equilibration to occur during each phase before cooling. In freezing these embryo suspensions, tedious programmed rates of cooling, ice nucleation induction and further cooling prior to storage is necessary. Recovery of embryos from storage requires reversing medium induction protocols allowing stepwise dilution of cryoprotectants. Presently optimal yields are obtained using expensive computer controlled rate freezers, cooling embryos at any desired increment of degree per unit of time. The disadvantage of this type of programmed cryopreservation is that it requires an expensive, bulky freezer units. Recent research has therefore focused on eliminating the need for controlled rate freezing by allowing embryos to be cryopreserved by direct transfer into liquid nitrogen (LN_2). As these techniques were first developed, step-wise addition/equilibration of embryos was required, but rapid freezing was induced by direct submersion of suspensions into LN_2. Slow and/or rapid recovery and dilution would then ensue.

Beginning in about 1985, vitrification techniques were developed in an attempt to limit cell damage due to crystallization. Vitrification permits cooling of embryos to -196°C without the

formation of ice crystals in the suspension, as reported by Rall and Fahy, Nature (London) 313, 573-575 (1985). Vitrification refers to a physical process by which a concentrated solution of cryoprotectants solidifies during ultra-rapid cooling to form a solidified "glass", retaining the normal molecular and ionic distributions of the liquid state in an extremely viscous, supercooled form. Survival of embryos in a vitrified state has been proven *in vitro*, as well as *in vivo*, by Rall and Fahy (1985) and Rall, Cryobiology 23, 548 (1986) and Cryobiology 24, 387-402 (1987), who also demonstrated in the latter article development into normal offspring of frozen embryos when warmed rapidly and transferred into the uteri of foster mothers.

There are basically two types of cryoprotectants. Permeating agents include dimethylsulfoxide (DMSO), glycerol, acetamide and 1,2-propanediol (propylene glycol, PG). Non-permeating agents include sucrose and polyethylene glycol (PEG). Cryoprotectants allow considerable supercooling. They act to depress the homogenous nucleation temperature, T_H, and raise the glass transition temperature of a given suspension, T_G. The concentration of cryoprotectants at a point when $T_H = T_G$ has been found to allow organ-sized samples to achieve complete transition into the glass phase without any sign of crystallization occurring. Not only does this allow escape from mechanical injury caused by ice formation, but it places sole responsibility of cellular damage on cryoprotectant toxicity. Cryoprotectant toxicity is reduced by reducing the concentration required for vitrification and by selection of compound or mixtures of compounds which enhance glass-forming ability and/or decrease toxicity. Cellular toxicity might also be reduced by incorporating additional solutes into the cryoprotectant mixture and/or shortening the equilibration time.

Vitrified embryos may still be highly susceptible to intracellular damage during recovery. If improperly thawed, the embryo suspension may undergo devitrification. This is a process by

which the metastable glass forms a stable crystalline phase or phases above its glass transition temperature, the direct result of not cooling at a sufficient rate. This condition can lyse the embryos. If water or straight saline were used to dilute out the cryoprotectants, the water would enter the cells too rapidly, causing swelling as the osmotic equilibrium is restored. Expansion of greater than 30% of normal embryonic size can cause membrane disruption and lysis. Two methods have been used to prevent osmotic damage: dilution of the vitrification solution in a stepwise manner and osmotic dilution of the vitrification solution using sucrose, an impermeable solute.

The frozen embryos are conventionally stored in French artificial insemination straws. As described by Scheffen, et al., Cryo-Lett 7, 260-269 (1986), embryos in vitrification medium can be stored in a straw with dilution medium, separated during storage by an air barrier. Upon thawing, the dilution medium is induced to flow around the embryo. Unfortunately, the dilution medium often causes cracking of the straw and can cause heterogenous mixing of straw contents upon warming.

Since all embryos must undergo equilibration, vitrification, thawing, dilution and rehydration without experiencing osmotic or toxic shock and devitrification, the solutions and methods, as well as the embryonic stage of development, are critical to survival of the cryopreserved embryos.

It is therefore an object of the present invention to provide a method and composition to cryopreserve embryos quickly, easily and efficiently without the use of a programmable freezer.

It is a further object of the present invention to provide a method and composition of vitrification of embryos which can be recovered with a high survival rate.

It is a still further object of the invention to provide a method and composition to vitrify embryos which does not cause

cracking of the storage straws, as well as to maintain homogeneity of solutions within the straws.

Summary of the Invention

A cryopreservation procedure that yields high percentages of normal births after rapid thawing of frozen embryos and implantation into recipient females. The techniques have been successfully used to cryopreserve mouse, rabbit and bovine embryos. *In vitro* success rates ranged from 75 to 90% survival to the mature blastocyst stage for mice and rabbits. *In vivo* rates for implantation and survival of embryos ranged from 15 to 30% and 40 to 50% for mice and rabbits, respectively.

Embryos are obtained by flushing the oviduct and uterus of donor females following prescribed periods post hCG using rehydration/recovery medium. These are equilibrated with equilibration medium for between five and ten minutes, preferably for seven minutes.

Artificial insemination straws are prepared containing layers of vitrification medium separated by air barriers, loaded with the embryos in as small a volume of equilibration medium as possible with additional vitrification medium added after the final air barrier. Sealant/identification rods are placed on the open end of the straws which are then vitrified by progressive immersion into liquid nitrogen.

Embryos are recovered by removing the straw from the liquid nitrogen and immediately immersing it into a room temperature water bath for five to ten seconds, unsealing both ends of the straw, and emptying the contents into a dish of dilution medium for five minutes. Following dilution, rehydration occurs when the embryos are placed into a volume of rehydration/recovery medium for fifteen minutes. After the proper incubation time, the embryos can be implanted into recipient females.

High yields are obtained by use of media all containing the same ion concentration, short equilibration times, and proper straw loading technique.

Brief Description of the Drawing

Figures 1A-1F are cross-sectional views of the straw loaded according to the present invention, actual size scale: 1A, the straw and syringe ready to be loaded; 1B, initial loading of VM for flooding wick; 1C, VM and air barrier loaded; 1D, VM, air barrier and VM for embryos loaded; 1E, VM, air barrier, and VM containing embryos loaded; and 1F, VM flooding wick and powder plug, air barrier, VM containing embryos, air barrier, VM contacting sealant rod, and sealant rod loaded and ready for immersion into liquid nitrogen.

Detailed Description of the Invention

The following procedure and compositions are used to cryopreserve 8, 16, and 32 cell morula or early blastocyst (non-expanded) stage embryos. Results may vary with the type and strain of the embryo. It is essential to examine embryos prior to cryopreservation since defective embryos should not be cryopreserved or transferred.

The techniques have been successfully used to cryopreserve mouse, rabbit and bovine embryos. *In vitro* success rates ranged from 75 to 90% survival to the mature blastocyst stage for mice and rabbits. *In vivo* rates for implantation and survival of embryos ranged from 15 to 30% and 40 to 45% for mice and rabbits, respectively.

The procedure described below is dependent on the use of the same base medium for each step of the procedure. Ion concentration is maintained throughout the procedure, varying only the osmotic strength as required for equilibration, vitrification, dilution and rehydration. The procedure is also highly dependent on the technique used in loading and freezing the straws. In the preferred

embodiment, 0.25 ml French straws, obtained from IMV International Corp., Minneapolis, MN, are used for storage of the embryos. A color coded identification rod approximately 50 mm in length is inserted into the loading end for identification and sealing purposes. A cotton wick and powder plug is used to seal the other end. Layers of vitrification medium, separated by barriers of air bubbles, are used to protect the embryo from contacting the cotton wick and the identification rod.

In the first step of the procedure, embryos are obtained by flushing the oviduct and uterus of superovulated donor animals at an appropriate time following administration of human chorionic hormone (hCG), 72 to 96 post hCG for mice, 60 to 90 hours for rabbits, using rehydration/recovery medium (RM). The embryos are then equilibrated with equilibrium medium for between five and ten minutes, preferably for seven minutes. Embryos are then washed twice with RM and morphologically incorrect or abnormal embryos discarded.

The formula for the rehydration medium is:

0.8% NaCl
0.02% KCl
0.01% $CaCl_2$
0.02% KH_2PO_4
0.0048% $MgCl_2$ $6H_2O$
0.115% Na_2HPO_4
0.0036% Na Pyruvate
0.1% glucose
0.3% bovine serum albumin
0.001% phenol red
100 IU penicillin/ml
50 μg streptomycin/ml

Equilibration in equilibration medium (EM) permits cellular dehydration and non-toxic cryoprotectant permeation to occur. The embryos are placed into a dish, such as a Falcon 1008 petri dish, containing a small volume, approximately 2.5 ml, EM. A short

equilibration time improves yield, preferably between five and ten minutes, most preferably seven minutes.

The equilibration medium consists of 10% glycerol and 20% propylene glycol in RM.

Following equilibration, embryos are placed directly into vitrification medium in prepared straws. Vitrification is a physical process by which a concentrated solution of cryoprotectants solidifies during ultra-rapid cooling to form a "glass", an extremely viscous supercooled liquid. A "glass" forms in transition from liquid to solid in the absence of ice crystallization, retaining the normal molecular and ionic distributions of a liquid state.

The embryos are loaded into the straws for vitrification. After proper loading, the straws are immersed into liquid nitrogen for storage. It is extremely important for straw and embryo loading procedures to be followed precisely. Poor loading technique can result in air bubble formation throughout the suspension within the straw, causing uneven cooling rates during exposure to liquid nitrogen. Care should be given during loading to maintain homogeneity of the vitrification medium within the straw to insure optimal survival rates. Upon immersion into liquid nitrogen, glass formation is induced throughout the vitrification medium. The vitrification medium should appear transparent in the straw, solidified in the glass state without ice crystallization.

Straws are prepared while embryos are equilibrating. The straw loading steps are shown schematically in Figures 1A through 1F. A syringe 10, for example, a Becton-Dickinson three cc syringe, is connected to the end of the straw 12 containing the wick 14 and powder plug 15. Vitrification medium (VM) 16 is drawn into the straw 12 to the 25 mm line pre-marked on the straw 18, which will provide a protective layer over the cotton wick 14. Next, air is drawn into the straw 12 to form a separation barrier 20. Finally, VM 22 is drawn into the straw to the line. Following equilibration, the embryos

24, with a minimal amount of EM, are then transferred into this layer of VM 22 using a prefilled pipette 23 containing a small, approximately 20 microliter volume of VM. An air barrier 26 is then drawn into the straw 12 to the line and a buffer of VM 28 to the point that the cotton wick 14 and powder plug 15 seals and the identification rod 30 can be inserted to directly contact the VM. Straws should not be bent.

The formula for the VM is 25% glycerol and 25% propylene glycol in RM.

The embryos are cryopreserved by immersing the straw into liquid nitrogen. Vitrified straws are stored in goblets on racks immersed in liquid nitrogen, for example, Nasco Shur-bend Model L goblets and Nasco Model DDS goblet racks.

To thaw vitrified embryos, straws are removed from liquid nitrogen storage and are warmed in a room temperature water bath (25°C). It is crucial that the straw is immediately submerged into the water bath to insure that a rapid warming rate is established. If straws are not immediately placed into the warming bath, a brief period of ice crystallization or devitrification could occur. Devitrification, which results from not warming at a sufficient rate, is visualized as a brief, two to three seconds, whitening of the solution during warming, and is extremely detrimental to the embryos.

After a few seconds, the glass transforms into the liquid phase. The embryos are removed from the straw directly into dilution medium by removing the identification rod and cutting the straw directly below the wick and powder plug. Contents are emptied into a small volume, approximately two ml, of dilution medium (DM) in a petri dish. The straw can also be rinsed with approximately 50 to 100 μl DM.

The formula for DM is 1 M sucrose in RM. During dilution, a gradient is established to pull the cryoprotectants out of the cells. Once dilution is complete, after a period of five minutes, the embryos are isotonically rehydrated into their normal state. The

embryos are then transferred from DM into RM and allowed to stand at room temperature for fifteen minutes. Embryos are then fully able to be transferred into recipient females for development into offspring.

Example 1: Effect on survival rates of short term 4°C storage of cryopreserved embryos.

The precursive effects of 4°C, short-term storage on survival of cryopreserved mouse embryos were determined. Embryos were obtained from superovulated random bred Swiss mice 68-70 h post-mating (compacting 8-16 cell stage). A two-step vitrification methodology was adapted utilizing glycerol and propylene glycol as cryoprotectants, with sucrose dilution of cryoprotectants upon rapid warming. Treated embryos were washed 3x in RM, and cultured for 36 h at 37°C.

There was no difference ($p>.05$) between freshly flushed (non-4°C stored) embryos and embryos store for 24 h at 4°C (195/271 or 72.0%, 291/401 or 72.6%, respectively). As a percent of controls developing to mature blastocysts, 78.9% freshly flushed embryos developed post-thawing as opposed to 89.6% 4°C stored (24 h) embryos. After 4°C storage these early compacting embryos often undergo "decompaction", but will develop into mature blastocysts upon subsequent culture. There was a significant difference ($p<.05$) in the ability to develop in culture post-thawing between compacted (72.7%) and decompacted (53.0%) embryos. No difference ($p>.05$) was observed in the proportion of embryos developing to blastocysts between compacted 4°C (24 h stored) (72.7%) and freshly flushed, non-stored (72.0%) embryos. Extended 4°C storage exposure for 48 and 96 h proved significantly ($p<.05$) detrimental for post-thaw culture survival, measuring 13.3% and 12.5%, respectively. These results indicate that storage at 4°C for 24 h has no detrimental effects

upon successful cryosurvival and ultimate culture development of compacted 8-cell embryos.

Example 2: Comparison of survival rates of cryopreserved outbred and crossbred mouse embryos.

The purpose of this study was to compare development rates of embryos from outbred and crossbred strains of mice following vitrification. Embryos were obtained from superovulated outbred CD-1 and crossbred B_6D_2 (C57BL/6J X DBA/2J) mice 68-70 h post-mating at the compacting 8-16 cell stage. Embryos to be vitrified were equilibrated for 7 min at 25°C in EM (RM containing 10% glycerol, 20% propylene glycol) and then loaded into 25 μl of VM (RM with 25% glycerol, 25% propylene glycol) in 0.25 ml plastic straws. The remaining area within the straws was filled with DM (RM with 1 M sucrose) separated from the VM by 10 μl air bubbles. Straws were immediately plunged into liquid nitrogen (LN_2 treatment group). For thawing, straws were submerged into a 25°C water bath for 5-10 sec. Straw contents were mixed by flicking the straw like a clinical thermometer 3-4X, then expelling the contents into 2 ml of fresh DM. After a 5 min incubation to remove intracellular cryoprotectants, embryos were allowed to rehydrate in 2 ml RM and then cultured for 24 h at 37°C in 7% CO_2 and 100% humidity.

Following culture, 74.4% (288/372) of the vitrified CD-1 embryos developed to expanded blastocysts compared with 90% (45/50, $p<.05$) for cultured controls. When CD-1 embryos were exposed to VM but not vitrified, the results (95.3%, 81/85) were also higher ($p<.05$) than those of vitrified embryos. For vitrified B_6D_2 embryos, 89.8% (219/244) developed to expanded blastocysts in culture compared to 94.6% (70/74, $p>.01$) of cultured controls. When B_6D_2 embryos were exposed to VM but not vitrified, 89.3% (184/206) of the embryos went on to develop which was not different ($p>.01$) from B_6D_2 culture control or vitrified embryos. B_6D_2 embryos

had a higher *in vitro* development rate ($p<.01$) following vitrification than CD-1 embryos. Of 89 CD-1 embryos vitrified, 15 normal fetuses (17% of the total) were produced after transfer to the oviducts of pseudopregnant recipients. These results indicate that vitrified embryos from crossbred strains of mice have a higher embryonic development rate than their outbred counterparts and these vitrified embryos will go on to develop into normal offspring.

Example 3: Cryopreservation of vitrified rabbit embryos.

The objective of this study was to develop a straightforward, reliable and inexpensive system of cryopreserving rabbit embryos. Embryos were obtained from superovulated, random bred does 60 h post-mating/hCG (75 IU) at the 32-cell, compacted morulae stage. Embryos to be vitrified were dehydrated and equilibrated for 7 min at 25°C in EM (RM containing 10% glycerol (G), 20% propylene glycol (PG)) and then loaded into 25 μl of VM (RM with 25% G, 25% PG) in 0.25 ml straws. The remaining area within the straws was filled with DM (RM with 1 M sucrose) separated from the VM by 10 μl air bubbles. Straws were then progressively plunged into liquid nitrogen (LN_2 treatment group). For thawing, straws were submerged into 25°C water bath for 5-10 sec. The straw contents were expelled into 2 ml of fresh DM in which a 5 min incubation was allowed to remove intracellular cryoprotectants.

Following dilution, the embryos were rehydrated in 2 ml RM for 15 min and then allocated to *in vitro* or *in vivo* groups. Cultured embryos were incubated for 48 h at 37°C in 7% CO_2 and 100% humidity. Embryos to be transferred were thawed and rehydrated accordingly then deposited into the oviduct of a pseudopregnant recipient. Control embryos were transferred into the contralateral oviduct of each recipient.

Following culture, 85% (85/100) of the vitrified embryos developed to mature (expanded or hatched) blastocysts compared with

91% (103/113, p>.10) for cultured controls. *In vivo*, 39% (16/41) of the surviving vitrified embryos implanted as compared to 24% (9/37,p>.10) of transplant controls. Of pregnant (5/6, 83%) females, 49% (16/33) of vitrified embryos developed to implantation as did 29% (9/31, p>.10) of transplant controls. Of these, 30% (13/33) of the vitrified embryos developed into normal offspring (day 26 after transfer) as did 23% (7/31, p>.10) of transplant controls. The results indicate that vitrification is a simple and reliable procedure for cryopreserving rabbit embryos. Vitrified embryos readily progress in culture and develop at high rates into normal offspring.

Example 4: Comparison of results obtained with inclusion of DM in straw versus inclusion of protective layers of VM.

Including DM, containing approximately 34% sucrose, in the straw loading regimen frequently causes cracking of the straws upon ultra-rapid cooling. This problem was overcome by allowing only the "glass" forming VM to be exposed within the straw. All straws are labelled at 25 mm from the open end of the straw. During the loading regimen, VM and air barriers are drawn in to this mark, providing optimal cryosurvival without damage to the straws.

Example 5: Cryopreservation of Bovine Embryos.

Preliminary studies indicated that the method and compositions of cryopreservation are successful with 32-cell to early blastocyst (non-expanded) bovine embryos. Development to mature blastocyst stage has been demonstrated *in vitro*, with *in vivo* data pending.

I claim.

1. A method for cryopreserving embryos by vitrification comprising

flushing embryos from the reproductive organs of donor females in an isotonic medium containing K^+, Na^+, Cl^-, and Mg^{+2} ions, Na pyruvate and glucose as energy sources, antibiotics, and an approximately physiological level of albumin;

equilibrating the embryos in equilibration solution of the isotonic medium containing 10% glycerol and 20% propylene glycol for between five and ten minutes;

loading a long slender tube formed of an inert material, having a capacity of approximately 0.25 ml, and plugged at one end with a volume of vitrifying solution of isotonic medium containing 25% glycerol and 25% propylene glycol, an air barrier, vitrifying solution, the embryos to be vitrified suspended in a minimal volume of the equilibration solution, an air barrier and vitrifying solution to the end of the straw, and a seal; and

immersing the loaded straw in liquid nitrogen or an equivalent thereof.

2. The method of claim 1 further comprising thawing the embryo by immersion of the straw into a room temperature bath until thawed.

3. The method of claim 2 wherein the straw is immersed for five to ten seconds.

4. The method of claim 2 further comprising recovering the embryo by removing the seal and releasing the embryo into dilution solution of isotonic medium containing 1 M sucrose for five minutes.

5. The method of claim 4 further comprising preparing the embryos for implantation comprising rehydrating the embryos for at least fifteen minutes in isotonic medium.

6. The method of claim 1 wherein the embryos are equilibrated for seven minutes.

7. The method of claim 1 wherein the isotonic medium has the composition: 0.8% NaCl, 0.02% KCl, 0.01% $CaCl_2$, 0.02% KH_2PO_4, 0.0048% $MgCl_2$ $6H_2O$, 0.115% Na_2HPO_4, 0.0036% Na Pyruvate, 0.1% glucose, 0.3% bovine serum albumin, 0.001% phenol red, 100 IU penicillin/ml, and 50 μg streptomycin/ml.

8. A composition for cryopreservation of embryos by vitrification comprising four solutions: an isotonic medium containing K^+, Na^+, Cl^-, and Mg^{+2} ions, Na pyruvate and glucose as energy sources, antibiotics, and an approximately physiological level of albumin; a vitrifying solution of isotonic medium containing 25% glycerol and 25% propylene glycol; an equilibrating solution of isotonic medium containing 10% glycerol and 20% propylene glycol; and a diluting solution of isotonic medium containing 1 M sucrose.

9. A cryopreserved embryo in a long slender tube formed of an inert material, having a capacity of approximately 0.25 ml, and plugged at one end with a volume of vitrifying solution of isotonic medium containing 25% glycerol and 25% propylene glycol, an air barrier, vitrifying solution, the embryo to be vitrified suspended in a minimal volume of the equilibrating solution, an air barrier and vitrifying solution to the end of the straw, and a seal.

10. The cryopreserved embryo of claim 9 wherein the isotonic medium has the composition: 0.8% NaCl, 0.02% KCl, 0.01% $CaCl_2$, 0.02% KH_2PO_4, 0.0048% $MgCl_2$ $6H_2O$, 0.115% Na_2HPO_4, 0.0036% Na Pyruvate, 0.1% glucose, 0.3% bovine serum albumin, 0.001% phenol red, 100 IU penicillin/ml, and 50 μg streptomycin/ml.

11. The cryopreserved embryo of claim 9 comprising at least one embryo selected from the group consisting of rabbit embryos, mouse embryos, and bovine embryos.

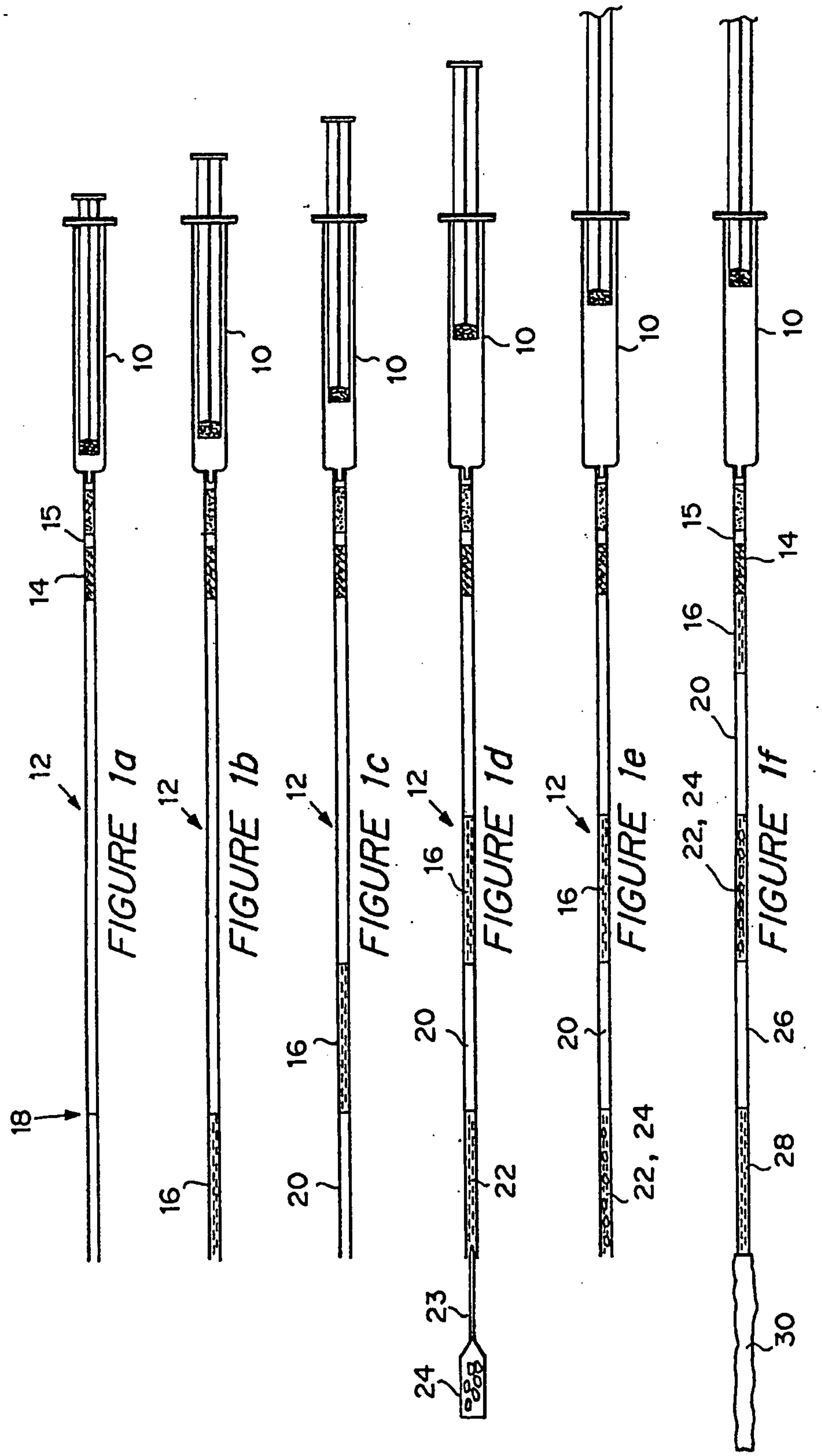
FIGURE 1a
FIGURE 1b
FIGURE 1c
FIGURE 1d
FIGURE 1e
FIGURE 1f
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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05402

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^5: A 01 N 1/02, A 61 K 35/54

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^5	A 01 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Indian Journal of Experimental Biology, volume 27, April 1989, K.P. Agrawal et al.: "Cryopreservation of mouse embryos at minus 196°C vitrification", pages 383-384 ---	
A	In Vivo, volume 2, 1988, (Athens, GR) F. Dagnaes-Hansen et al.: "Quick Freezing of mouse embryos: Freezing of inbred strains and 2-and 4-cell embryos by vitrification", pages 339-344 ---	
A	Am. Med. Vét., volume 131, 1987, A. Massip et al.: "Cryoconservation de l'embryon bovin: techniques et résultats" pages 515-528 ---	

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	24 JAN 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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A	Nature, volume 313, no. 6003, 14-20 February 1985, W.F. Rall et al.: "Ice-free cryopreservation of mouse embryos at minus 196°C by vitrification", pages 573-575	